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PRINCIPAL INVESTIGATOR: Rajeev S. Samant, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
                            Birmingham, Alabama   35291-0111

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6. AUTHOR(S)
Rajeev S. Samant, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35291-0111

E-Mail: rsamant@path.uab.edu

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13. ABSTRACT (Maximum 200 Words)

The focus of this study is to understand the biology behind the metastasis suppression via BRMS1, a recently identified metastasis suppressor gene. BRMS1 is a protein with a glutamic acid rich N-terminus, coiled-coil domain, an imperfect leucine zipper and nuclear localization signals. It is expressed almost ubiquitously in human tissues and is highly conserved across species. Sub-cellular fractionation and fluorescence immuno-cytochemistry has indicated that it localizes to nucleus. BRMS1 is shown to restore homotypic gap-junctional communication. Our hypothesis is that it may be involved in transcription regulatory complex.

To identify proteins that interacting with BRMS1 a yeast two-hybrid screen was performed using full length BRMS1 as a bait and human mammary gland library as a prey. We confirmed RBP1 (Rb binding protein), FLJ00052 (EST), MRJ (Hsp40 related chaperon) and Nmi (N-myc interactor) as potential interactors at cellular level by co-immunoprecipitation studies. We have further demonstrated that BRMS1 is a component of mSin3-HDAC complex. Based on these observations it is tempting to speculate that BRMS1 regulates gene expression by histone deacetylation. Currently we are studying the role of this complex in regulation of metastasis of breast cancer.

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The proposed work comprises a single specific aim of mutagenizing Breast Cancer Metastasis Suppressor 1, BRMS1, for establishing it's mechanism of action. The basic work is broadly divided into two parts (a) Mutational analysis (b) Identification of interacting protein(s).

The mutational analysis involves
- Constructing BRMS1 deleted for predicted domains and testing the effect of deletions *in vitro* and *in vivo*.
- Construction of site directed mutations and testing them *in vivo* and *in vitro*.

Identification of protein(s) interacting with BRMS1 involves
- Screening of "prey" library to identify the possible interactors.
- Test the effect of critical mutations identified by mutational analysis on the protein-protein interaction.

Both these parts were proposed to be carried out simultaneously and in this report the progress on both fronts is summarized.

Until June 2002 the project had following Key Accomplishments :

✓ Yeast two hybrid screen for protein interactors of BRMS1 is successfully performed
✓ Eight genetic interactors were discovered.
✓ RBP1 and MRJ interactions were verified with co-immuno precipitation
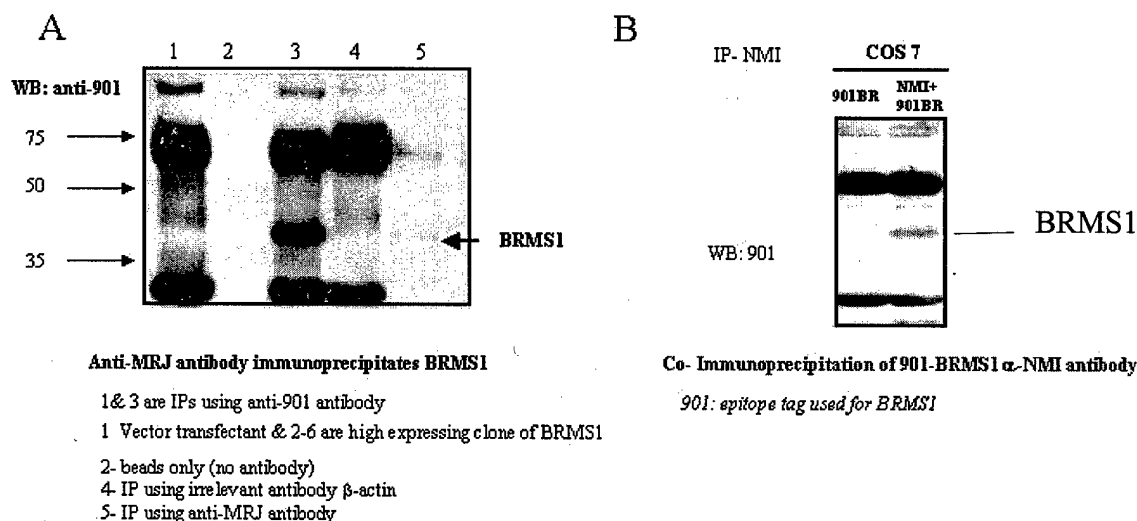✓ Assays of in vitro characterization of BRMS1 were standardized.

Following advice from Judy Pawlus (Technical Editor, Office of the Deputy Chief of Staff for Information Management), I would like to bring it to the notice of the reviewers that my mentor Prof. Danny Welch has moved the laboratory from The Pennsylvania State University to The University of Alabama at Birmingham (UAB). This move resulted in a period of less work from October 2002 till December 2002. We must emphasize that there is a substantial progress in this project and we are accomplishing to the initially proposed work schedule. Also effective June 2003, I have transitioned to a non-tenure track faculty position at the Department of Pathology, UAB and continuing work on this project for the completion.

Summary of the work:

Identification of interactors of BRMS1

Prey libraries from three different human tissues *viz.* breast, placenta and prostate were screened. The breast library was chosen based on the fact that BRMS1 was identified based on studies on metastatic breast carcinoma cell liens and was functionally shown to block the metastasis of breast cancer cell lines in nude mouse model. Placenta and prostate are tissues that express the highest levels of BRMS1. The screen was performed using full length BRMS1 as a bait. The results of these screens are summarized in Table1 (numbers indicate independent

clones). Eight genetic interactors of BRMS1 were identified. These are RBP1 (Rb binding protein), MRJ (Hsp40 related chaperone), CCG1 (a protein essential for progression of G phase), SMTN (cytoskeletal protein specific to smooth muscles), FLJ00052 (EST), KPNA5 (karyopherin alpha 5), Nmi (N-myc interactor), and BAF 57(BRG1 associated factor). The BRMS1-RBP1 (please refer to attached manuscript), BRMS1-MRJ as well as BRMS1-NMI interactions were further confirmed at cellular level by co-immunoprecipitation studies (Figure 1 a, b)

A

WB: anti-901

Anti-MRJ antibody immunoprecipitates BRMS1

1 & 3 are IPs using anti-901 antibody
1 Vector transfectant & 2-6 are high expressing clone of BRMS1

2- beads only (no antibody)
4- IP using irrelevant antibody β-actin
5- IP using anti-MRJ antibody

B

IP- NMI

WB: 901

Co- Immunoprecipitation of 901-BRMS1 α-NMI antibody

901: epitope tag used for BRMS1

Further characterization of the immunoprecipitated complex using HPLC, co-immunoprecipitation and Western blotting, suggests BRMS1 is a member of mSin3-HDAC complex. [Manuscript submitted to *Cancer Cell* is attached for a detailed description of this work].

Interestingly the EST, FLJ00052, discovered by us in the yeast two hybrid screen as coding for a possible interactor for BRMS1 is published recently as mSDS3 or SAP45 (Fleischer *et al,* Alland *et al*). mSDS3 is also shown to be a member of mSin3-HDAC complex . We have confirmed the BRMS1-mSDS3 interactions using the antibodies kindly provided by Don Ayer Huntsman Cancer Institute Department of Oncological Sciences, Salt Lake City, UT. (Fig 2 a & b).
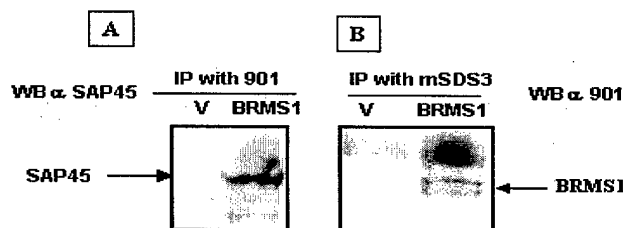

Fig 2. A: Immunoprecipitation of 901 tagged BRMS1 also pulls down Msds3
    B: Immunoprecipitation of mSDS3 pulls down Msds3
    These studies were carried out in 901 tagged BRMS1 expressors of MDA-MB-231

mSDS3 shares 24 %identity and 50% similarity with BRMS1. Based on GenBank searches performed by us and others MGC11296 is another EST that is possibly coding for a stronger ~60 % homolog of BRMS1. Thus we have found a family of BRMS1-proteins.

Our finding that BRMS1 interacts with mSDS3 and is a part of the mSin3-HDAC complex combined with other groups finding that mSDS3 forms a part of the same complex imply strongly that BRMS1 and mSDS3 are present together in the same complex. It is intriguing to see the BRMS1 family (homologs) are present in a complex that regulates gene expression. Based on the literature search, there are no major findings reported yet regarding which downstream genes are regulated by this complex. But it is tempting to speculate that this is a complex that plays a major role in control of the metastatic phenotype. Experiments are underway to address this question.

We had proposed that we will perform BRMS1 mutagenesis to see loss of the metastasis suppression function. We have decided to use this strategy in a slightly modified way. We will first find the mutant(s) that abrogates the participation of BRMS1 in the mSin3-HDAC complex. This mutant(s) will then be evaluated for loss of metastasis suppression ability. The mutants generated are described below.

Mutational analysis:

We had previously inspected BRMS1 for conserved domains and found various domains such as coiled-coil domain, nuclear localization sequences, N-terminal glutamic acid rich region and an imperfect leucine zipper. We looked at the BRMS1 protein sequence again in the light of the Y2H results, using the Pfam conserved domain search provided by NCBI. Many interesting conserved domains were observed. Many of them overlap and are disrupted in the BRMS1 deletion performed by us as explained below. We have used the ExSite™ inverse PCR strategy to create specific internal deletions of BRMS1.

- Deletion of Glutamic acid rich region   BRMS1ΔE: deletion of aa.11 to 63
- Deletion of Coiled coil domain   BRMS1 ΔC: deletion of aa. 67 to 87
- Deletion of Leucine zipper region   BRMS1 ΔL: deletion of aa. 138 to 181

We have also independently mutated the two NLS sequences to alanine and also have both the NLS mutated to alanine. All these constructs have N-terminal c-Myc tag for the ease of performing co-immunoprecipitations.

We are also using yeast reverse two hybrid screen using RBP1 as a bait to obtain mutated BRMS1 that would fail to interact. Both the approaches are expected to yield specific amino acids required for BRMS1 to be a member of the mSIN3-HDAC complex. These mutants will then be evaluated in animals for metastasis suppression. If the formation of the complex is relevant to metastasis suppression, mutated BRMS1 will lose that capability.

Key Accomplishments in 2002-2003:

- BRMS1 interaction with NMI confirmed
- BRMS1 mSDS3 interaction confirmed
- Discovered BRMS1 as one of the member of a ~1.6 MDa mSin3-HDAC complex.
- Generated specific internal deletions in BRMS1 to disrupt various domains.
- Submission of manuscript to *Cancer Cell.*

Reportable Outcomes:

Publications in Peer reviewed journals

1. **Samant, R.S.**, Seraj, M.J., Saunders, M.M., Sakamaki, T., Shevde L.A., Harms, J.F., Leonard, T.O., Goldberg S.F., Budgeon, L., Meehan, W.J., Winter, C.R., Christensen, N.D., Verderame, M.F., Donahue, H.J., and Welch, D.R. Analysis of mechanisms underlying BRMS1 suppression of metastasis. *Clin Exp Metastas.* **18** (8): 683-693 (2001)

2. Manni, A., Washington, S., Griffith, J.W., Verderame, M.F., Mauger, D., Demers, L.M., **Samant, R.S.**, and Welch D. R. Polyamine involvement in invasion and metastasis by human breast carcinoma cells. *Clinical and Experimental Metastasis*, **19** (2), 95-105 (2002)

3. Shevde LA, **Samant RS**, Goldberg SF, Sikaneta T, Alessandrini A, Donahue HJ, Mauger DT, Welch DR. Suppression of Human Melanoma Metastasis by the Metastasis Suppressor Gene, BRMS1. *Exp Cell Res*: **273**(2), 229-239 (2002)

4. Park Y.G., Lukes L., Yang H., Debies M.T., **Samant R.S.**, Welch D.R., Lee M., Hunter K.W. Comparative sequence Analysis in Eight Inbred Strains of the Metastasis Modifier QTL Candidate Gene *Brms1. Mammalian Genome*, **13** (6), 289-292.

5. **Samant, R.S.**, Debies, M.T., Shevde, L.A, Verderame, M.F., and Welch, D.R. Identification and characterization of murine ortholog (*brms1*) of Breast Cancer Metastasis Suppressor 1 (*BRMS1*). *Int. J. Cancer*: **97**, 15-20 (2002).

6. **Samant, R.S***; Meehan, WJ.*,., Hopper, JE., Workman, JL., Carrozza, MJ., Shevde, LS., Verderame, MF., Eckert KE., Welch , DR. Interaction of the BRMS1 metastasis suppressor with RBP1 and mSin3 histone deacetylase complex.**(Manuscript submitted to *Cancer Cell)***

7. Cicek, M., **Samant, R. S.**, Kinter, M., Welch, D.R., and Casey, G. Identification of metastasis associated proteins through protein analysis of Metastatic Breast Cancer Cell line MDA-MB-435 and Metastasis Suppressed BRMS1 transfected MDA-MB-435. **(Manuscript submitted to *Clinical and Experimental Metastasis*)**

Abstracts

1. Shevde LA, **Samant RS**, Casey G and Welch DR. BRMS1 suppresses expression of osteopontin.*9th international congress of the Metastasis Research Society*, Chicago, September 20-22, 2002.
2. **Samant RS**, Meehan WJ, Shevde LA, Hopper JE, Welch DR. Identifying protein interactors of Breast Cancer Metastasis Suppressor 1, BRMS1. *9th international congress of the Metastasis Research Society*, Chicago, September 20-22, 2002.
3. **Samant RS**, Meehan WJ, Shevde LA, Hopper JE, Welch DR. Identifying protein interactors of Breast Cancer Metastasis Suppressor 1, BRMS1. *Era of Hope*, 2002. CDMRP meeting of the U.S. Army Materiel Command, Florida, September 24-28, 2002.
4. Welch, D.R., Harms, J.F., **Samant, R.S.**, Babu, G.R., Gay, C.V., Mastero, A.M., Donahue, H.J.,Griggs, D.W., Kotyk, J.J., Pagel, M.D., Rader, R.K., Westlin, W.F., The small molecule $\alpha v \beta 3$ antagonist (S247) inhibits MDA-MB-435 breast cancer metastasis to bone. *3rd North American Symposium on Skeletal Complications of Malignancy*. 3:A17, 2002 .
5. Li, Z., Zhou, Z., **Samant, R.S.,** Babu, G.R., Kapoor, P., Welch, D.R. and Donnahue, H.J. Connexin 43 expression suppresses breast cancer cell tumorigenicity and metastasis. *Proceedings of the American Association for Cancer Reaearch* (2003).
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The BRMS1 metastasis suppressor forms complexes with RBP1 and the mSin3 histone deacetylase complex and represses transcription

William J. Meehan [a,b], Rajeev S. Samant [a,b,g], James E. Hopper [c], Michael J. Carrozza [e], Lalita A. Shevde [b,g], Jerry L. Workman [e], Kristin A. Eckert [b,c], Michael F. Verderame [d], and Danny R. Welch [b,f,g,h,*]

[a] these authors contributed equally to this work

[b] Jake Gittlen Cancer Research Institute, Department of Pathology; [c] Department of Biochemistry; [d] Department of Medicine, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033-0850;

[e] Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110;

[f] National Foundation for Cancer Research Center for Metastasis Research; [g] Department of Pathology, [h] Comprehensive Cancer Center, University of Alabama at Birmingham, 1670 University Blvd. Volker Hall G-038, Birmingham, Alabama 35294-0019

[*] Corresponding author:

        Danny R. Welch, Ph.D.
        Leonard H. Robinson Professor of Pathology
        Department of Pathology
        University of Alabama at Birmingham
        1670 University Blvd.
        Volker Hall G-038
        Birmingham, Alabama 35294-0019
        Tel.:   205-934-2956
        Fax: 205-934-1775
        E-mail:dwelch@path.uab.edu

Running title: BRMS1 interacts with the mSin3 HDAC complex

Summary

Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis of multiple human and murine cancer cells without inhibiting tumorigenicity. By yeast two-hybrid and co-immunoprecipitation, BRMS1 interacts with retinoblastoma binding protein 1 (RBP1) and at least seven members of the mSin3 histone deacetylase (HDAC) complex in human breast and melanoma cell lines. BRMS1 co-immunoprecipitates enzymatically active HDAC proteins and represses transcription when recruited to a Gal4 promoter *in vivo*. BRMS1 exists in large mSin3 complex(es) of ~1.4 to 1.9 MDa, but also forms smaller complexes with HDAC1. Deletion analyses show that the carboxyl terminal 42 amino acids of BRMS1 are not critical for interaction with much of the mSin3 complex, and that BRMS1 appears to have more than one binding point to the complex. These results further show that BRMS1 may participate in transcriptional regulation *via* interaction with the mSin3:HDAC complex and suggest a novel mechanism by which BRMS1 might suppress cancer metastasis.

Significance

Discovery of metastasis suppressor genes continues at a rapid rate but the mechanisms by which they control metastasis have remained relatively enigmatic. The present study identifies a transcriptional complex involving the metastasis suppressor BRMS1, mSin3 and histone deacetylases 1 and 2. The results further imply that regulation of specific histone deacetylases may be important for controlling cancer metastasis.

Introduction

The complex process of cancer cell dissemination and establishment of secondary foci involves the acquisition of multiple abilities by metastatic cells. For example, blood-borne metastasis requires cells to invade from the primary tumor, enter the circulation, survive transport, arrest at a secondary site, recruit a blood supply and proliferate at that site (Fidler 1990; Fidler and Ellis 1994). The ability to accomplish all of these steps likely involves changes in, and coordinated expression of, a large assortment of genes. Consistent with this notion, several genes, proteins and pathways have been associated with metastatic progression, including oncogenes, motility factors, and matrix metalloproteinases (Fidler 1990; Fidler and Ellis 1994; Welch and Wei

1998). In addition to metastasis-promoting genes, a new class of molecules called metastasis suppressors has been described [reviewed in (Steeg 2003; Shevde and Welch 2003). By definition, metastasis suppressors inhibit metastasis without blocking primary tumor growth, presumably by inhibiting one or more steps necessary for metastasis. To date thirteen metastasis suppressor genes have been identified that reduce the metastatic ability of cancer cell line(s) *in vivo* without affecting tumorigenicity: BRMS1, CRSP3, DRG1, KAI1, KISS1, MKK4, NM23, RhoGD12, RKIP, SseCKs, VDUP1, E-cadherin and TIMPs (reviewed in (Shevde and Welch 2003; Steeg 2003)).

We identified *BRMS1* (breast cancer metastasis suppressor 1) using differential display to compare highly metastatic breast carcinoma cells with related, but metastasis-suppressed cells (Seraj et al. 2000). Enforced expression of BRMS1 suppressed metastasis in three animal models – human breast (Seraj et al. 2000), murine mammary (Samant et al. 2002), and human melanoma cells (Shevde et al. 2002). Additionally, *BRMS1* mapped to loci in murine (LeVoyer et al. 2000; Hunter et al. 2001) and human (Seraj et al. 2000) genomes that had previously been implicated in metastasis control in breast carcinoma (Welch and Wei 1998). The BRMS1 protein localized to nuclei and restored gap junctional intercellular communication in both breast and melanoma tumor cell lines (Shevde et al. 2002; Saunders et al. 2001; Samant et al. 2001), but its molecular functions remain to be elucidated.

One approach to determine mechanism of action involves identifying which proteins interact with BRMS1. In this report, we utilized yeast two-hybrid and co-immunoprecipitation (co-IP) to demonstrate that BRMS1 interacts with retinoblastoma binding protein 1 (RBP1). This association led to experiments to demonstrate that BRMS1 interacts with at least seven members of the mammalian Sin3 (mSin3) mSin3:histone deacetylase (HDAC) complexes, including HDAC1 and HDAC2.

Human HDACs exist in many large, multi-subunit protein complexes (Ng and Bird 2000; Zhang et al. 1998) that are recruited to specific regions by DNA-binding factors. As their name indicates, HDACs remove acetyl groups from lysine residues at the amino-terminal tails of core histones (Knoepfler and Eisenman 1999; Ahringer 2000; de Ruijter et al. 2003). Histone deacetylation favors transcriptional repression, while acetylation (mediated by histone acetyltransferases) favors transcriptional activation. mSin3:HDAC complexes are named for the

large mSin3A and mSin3B proteins, which are thought to serve as scaffolds for complex assembly (Ahringer 2000). HDAC enzymatic activity in mSin3 complexes is mediated by a core subunit consisting of HDAC1, HDAC2, RbAp46, and RbAp48 (Knoepfler and Eisenman 1999). The core HDAC subunit is also found in at least one other HDAC complex, nucleosomal remodeling and deacetylation (NuRD) (Xue et al. 1998).

Mammalian Sds3 (mSds3) was recently reported to be an integral component of the mSin3 complex, and acts to stabilize HDAC1 within the complex (Alland et al. 2002). BRMS1 shares homology with mSds3, suggesting that BRMS1 belongs to a protein family (Alland et al. 2002). mSin3-associated proteins, SAP18 and SAP30, which are believed to serve as adapter molecules, complete the core complex as currently understood (Zhang et al. 1997; Laherty et al. 1998; Zhang et al. 1998).

Results

RBP1 and mSds3 were identified as BRMS1-interacting proteins by yeast two-hybrid screen

A yeast two hybrid screen was performed using prey libraries from three human tissues: breast, placenta and prostate. Breast was chosen because BRMS1 was first identified as a metastasis suppressor in breast cancer. Placenta and prostate were chosen because BRMS1 mRNA is highly expressed in these tissues (Seraj et al. 2000). Full-length BRMS1 was used as the 'bait'. RBP1 was present in the majority of positive clones from breast and placenta libraries, so it was chosen for further studies (Fig. 1A, 1B).

The FLJ00052 expressed tag was present as two independent positive clones in a prostate library screen. During the completion of the work reported here, FLJ00052 was identified as the mammalian ortholog (mSds3, GenBank Accession number XM 045014 mapping to human chromosome 12q24.23) of the yeast Sds3 protein. There are other related genes according to the LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=FLJ00052&ORG=&V=0), suggesting existence of additional mSds3 orthologs. mSds3 is an integral component of the mSin3:HDAC co-repressor complex, modulates HDAC activity, and stabilizes the complex (Alland et al. 2002). Antibodies recognizing mSds3 are not available commercially; so, we have not yet been able to test whether BRMS1 pulled down mSds3.

BRMS1 and RBP1 are reciprocally co-immunoprecipitated in human breast and melanoma cancer cells.

MDA-MB-231 human breast carcinoma cells and C8161.9 human melanoma cells were transfected with 901 epitope-tagged BRMS1. Immunoprecipitation (IP) of BRMS1 followed by immunoblot with two RBP1-specific antibodies (clones LY11 and LY32) (Fig. 1C, 1D) showed that BRMS1 co-IP RBP1 (Fig. 1C, 1D). Negative controls (co-IP using anti-901 in vector-transfected cells or co-IP using an irrelevant antibody (anti-Lamin A/C)) did not pull down RBP1 (Fig 1C, 1D). Antibody directed against RBP1 co-IP BRMS1 in both breast carcinoma (Fig. 1E) and melanoma (Fig. 1F) cells.

In order to begin defining the binding domains of BRMS1 responsible for interactions with RBP1, three C-terminal deletion mutants of 901-tagged BRMS1 were generated by exonuclease III digestion, designated BRMS1 (Δ204-246), BRMS1(Δ164-246), and BRMS1(Δ91-246) (Fig. 2C). Deletion constructs were transfected into both MDA-MB-231 and C8161.9. The latter expressing clones were experimentally more useful because expression of all three deletion mutants was approximately equivalent to full-length protein (data not shown, but can be inferred from Fig. 2B). In MDA-MB-231, only BRMS1(Δ204-246)-expressing clones had protein levels approximating full-length BRMS1 (inferred from Fig. 2A). Anti-901 antibody was used to co-IP deletion mutants and immunoblotting was used to detect RBP1 (Fig. 2A, 2B). Loss of amino acids 204-246 did not decrease binding to RBP1 in either cell line (Fig. 2A, 2B). Loss of aa 164-246 diminished binding (by ~90% by densitometry); and loss of aa 91-246 abrogated binding (Fig. 2B). Absence of binding by BRMS1 (Δ91-246) controlled internally for nonspecific binding of RBP1 to the 901 epitope. Interestingly, in both MDA-MB-231 and C8161.9, BRMS1 (Δ204-246) co-IP RBP1 more effectively (~1.5-fold) than full-length BRMS1 (Fig. 2A, 2B).

BRMS1 does not appear to complex with Rb or p107, nor to modulate E2F-dependent gene expression

RBP1 binds retinoblastoma (Rb) family members p105 (RB) and p107 (Fattaey et al. 1993; Lai et al. 1999; Lai et al. 2001). Rb proteins, in turn, bind E2F and tether RBP1 to E2F-responsive gene promoters. In this way, RBP1 directly suppresses transcription. We tested the hypothesis that BRMS1 is part of an RBP1:Rb:E2F complex; but, BRMS1 did not co-IP RB or p107 in

MDA-MB-231 (Fig. 2A) or C8161.9 cells (data not shown). Likewise, BRMS1 did not affect luciferase expression using an E2F-responsive promoter (data not shown). Taken together, these findings suggest that BRMS1 does not act as part of an RBP1:Rb:E2F complex and that BRMS1 might be part of a previously undescribed RBP1 complex that does not contain Rb.

BRMS1 co-IP several ^{35}S-labeled proteins in MDA-MB-231

Anti-901 was used to co-IP BRMS1 from ^{35}S-labeled lysates from BRMS1-transfected MDA-MB-231. Vector-transfected cells were used as controls. In addition to BRMS1, several additional bands were evident, including prominent large proteins at ≥200 kDa, ~160 kDa and ~65 kDa as well as less intense bands just below 50 kDa and another at ~30 kDa. (Fig. 3). Parallel experiments were done using *BRMS1*-transfected C8161.9 and *Brms1* (murine ortholog (Samant et al. 2002))-transfected 66cl4. Similar ^{35}S-labeled proteins were co-IP by anti-901 (data not shown). The pattern was reminiscent of previously published results showing that RBP1 interacts with the mSin3:HDAC complex (Lai et al. 1999; Lai et al. 2001). Specifically, HDAC1 and HDAC2 migrate at ~65/60 kDa. mSin3B and mSin3A migrate at ~160/150 kDa. These molecular weight proteins corresponded to the most prominent radiolabeled proteins co-IP with BRMS1 (Fig. 3). Therefore, we hypothesized that BRMS1 is a component of the mSin3:HDAC complex.

BRMS1 is a component of the mSin3:HDAC complex in C8161.9 and MDA-MB-231

IP of epitope-tagged BRMS1 followed by immunoblot showed that BRMS1 pulled down seven proteins previously shown to be part of mSin3:HDAC complexes — mSin3A, mSin3B, HDAC1, HDAC2, SAP30, RbAp46, and RbAp48 (Fig. 4). The same proteins were not precipitated in vector-transfected cells (Fig. 4, lane 1), nor were they pulled down using an antibody to the nuclear protein Lamin A/C (Fig. 4, lane 2). Western blots demonstrated that BRMS1-associated proteins were present at comparable levels in both vector- and BRMS1-transfected cell lysates (data not shown), ruling out the possibility that vector-transfected cells had lower levels of mSin3:HDAC complex components. Interactions between BRMS1 and mSin3:HDAC were relatively strong, since they persisted in 0.5 M NaCl. Antibodies recognizing mSin3B, HDAC1, HDAC2, and SAP30 "reverse" co-IP BRMS1 in C8161.9 cells as well (Fig. 6A).

mSin3:HDAC complex proteins exhibited the same general interaction pattern with BRMS1

deletion mutants as did RBP1, with some exceptions. BRMS1 (Δ204-246) co-IP mSin3A, mSin3B, SAP30, and HDAC2 at levels comparable to full-length BRMS1 (Fig. 4). However, BRMS1 (Δ204-246) co-IP HDAC1, RbAp46, and RbAp48 less efficiently than full-length BRMS1 (reduced ~40% by densitometry) (Fig. 4). This discrepancy is evident on co-IP/immunoblots simultaneously probed for HDAC1 and mSin3B, clearly demonstrating differential binding (data not shown). BRMS1 (Δ164-246) co-IP all mSin3:HDAC complex components significantly less efficiently than full-length BRMS1 (reduced ~90% by densitometry); whereas, BRMS1 (Δ91-246) did not co-IP any complex proteins (Fig. 4).

To determine if BRMS1 interacted with mSin3:HDAC complex proteins in human breast cancer cells, proteins were co-IP from BRMS1-transfected MDA-MB-231. Six mSin3:HDAC complex proteins — mSin3A, mSin3B, HDAC1, HDAC2, SAP30, and RbAp48 (Fig. 5) — were pulled down with BRMS1. Co-IP in vector-transfected cells did not co-IP these proteins (Fig. 5, lane 1) despite the proteins being present in both vector- and BRMS1-transfected lysates (Fig. 5, lanes 4,5). As above, interactions persisted in 0.5 M NaCl. RbAp46, a member of the core mSin3:HDAC complex, did not co-IP with BRMS1 in MDA-MB-231 cells (Fig. 5). Antibodies recognizing mSin3B, SAP30, HDAC1, and HDAC2 co-IP BRMS1 in MDA-MB-231 (Fig. 6B). BRMS1 (Δ204-246) co-IP mSin3:HDAC proteins at levels comparable to full-length BRMS1 (Fig. 5). In both melanoma and breast carcinoma cells, it was not possible to definitively demonstrate that BRMS1 co-IP SAP18, since SAP18 anti-sera also recognized a band at ~18 kDa in vector- and BRMS1-transfected cells (Fig. 5).

BRMS1 interacts with a subset of mSin3:HDAC complexes

Many proteins that bind HDAC complexes are responsible for recruiting complexes to specific DNA promoters. However, BRMS1 does not have a predicted DNA-binding motif, suggesting that it might serve a different role as a member of subsets of mSin3:HDAC complexes.

As a first step to evaluate those potential roles, the ability of BRMS1 to co-IP selected HDAC complex components was tested. Mad and Max were the first proteins shown to recruit the mSin3:HDAC to a specific promoter (Hassig et al. 1997; Laherty et al. 1997; Ayer et al. 1995), but BRMS1 did not co-IP Mad1 or Max (data not shown). The unliganded nuclear hormone co-receptors SMRT and NCoR have also been reported to recruit the mSin3 (Alland et al. 1997;

Heinzel et al. 1997; Nagy et al. 1997; Brehm et al. 1998), but there are contradictory data (Li et al. 2002a). In our system, BRMS1 did not co-IP SMRT or NCoR (data not shown). mSin3:HDAC interaction with MeCP2, a methyl CpG-binding protein, has also suggested that repression associated with DNA methylation may be mediated, in part, by deacetylation (Nan et al. 1998). Yet, BRMS1 did not co-IP MeCP2 (data not shown). Since the core HDAC subunit (HDAC1, HDAC2, RAp46, RbAp48) is also present in the NuRD HDAC complex (Xue et al. 1998), we asked whether BRMS1 complexed with NuRD. BRMS1 did not co-IP Mi-2 or MTA1, two members of the NuRD complex (data not shown). HDAC3, which is related to HDAC1 and HDAC2 and which can complex with RBP1 (Lai et al. 2001), did not co-IP with BRMS1 (data not shown).

Taken together, these data suggest that BRMS1 exists in a specialized subset of mSin3:HDAC complexes, rather than existing as an integral component of the complex. In other words, BRMS1 is not a ubiquitous member of mSin3:HDAC complexes.

BRMS1 exists in large (1.4 and 1.9 MDa) mSin3:HDAC complexes as well as smaller complexes containing HDAC1

In order to determine the size of BRMS1:mSin3:HDAC complex(es) and to determine the distribution of these molecules in complexes of various sizes, whole-cell protein lysates from C8161.9 were subjected to Superose 6 size exclusion chromatography. Fractions were separated by PAGE, transferred to PVDF, and immunoblotted for 901-BRMS1, HDAC1, SAP30, and mSin3B. These four proteins were chosen because they are core members of the complex. BRMS1 eluted in multiple peaks from the column with complex sizes ranging from ~ 100 to 2,000 kDa. BRMS1 elution was most prominent in peaks 5 and 6 (~1.7 MDa). HDAC1 also eluted in multiple peaks (fractions 4-22) with the majority present in fractions 8 and 9 (~ 1.4 MDa, Fig. 8A). SAP30 was detected in two peaks, one from fractions 4 through 14 and another from fractions 19-24, suggesting at least two complexes, the first >1.0 MDa and the second <200 kDa (Fig. 8A). mSin3B is detected uniformly in fractions 3-17, indicating involvement in complexes ranging from ~2.0 MDa to hundreds of kDa (Fig. 8A).

BRMS1 was immunoprecipitated from 420 µl of each fraction followed by PAGE and immunoblot. The vast majority (>90%) of BRMS1 was present in complexes ranging in size between 1.4 and 1.9 MDa (Fractions 5-9, Fig. 8B). BRMS1 also precipitated in fractions 10-23.

HDAC1, SAP30, and mSin3B co-IP with BRMS1 in fractions 5-9, although SAP30 is most abundant in fractions 8 and 9 (Fig. 8B). HDAC1, however, also co-IP with BRMS1 in fractions 10-21, suggesting that BRMS1 can be involved in smaller complexes with HDAC1 (Fig. 8B).

BRMS1 co-immunoprecipitates HDAC activity

To determine if BRMS1-associated HDAC1 and HDAC2 were enzymatically active, complexes were assessed for deacetylase activity in C8161.9. Full-length BRMS1 co-IP HDAC activity, BRMS1(Δ204-246) pulled down less HDAC activity. BRMS1(Δ164-246) co-IP still less HDAC activity, while BRMS1(Δ91-246) pulled down only background activity (Fig. 7). This pattern is reminiscent of the pattern of interaction with HDAC1 seen by immunoblot (Fig. 4). As a positive control, anti-HDAC1 antibodies were able to pull down HDAC activity (Fig. 7) proportionate to the amount of antibody used (i.e., when 2X anti-HDAC1 was used, double the HDAC activity was precipitated). These results show that only a small portion of the HDAC1 activity present in the protein lysate is being measured. Vector-transfected cells and co-IP with anti-Lamin A/C served as negative controls (Fig. 7).

BRMS1 represses transcription in luciferase reporter assays

On the basis of its physical interactions with mSin3 and HDAC1, it was predicted that BRMS1 would repress transcription. To investigate this, we measured the effect of BRMS1 on transcription using a luciferase reporter containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter. BRMS1 strongly repressed (~80%) basal transcription compared to the pBIND vector alone (Fig. 9).

Discussion

Epigenetic regulation of the metastatic phenotype was proposed in 1889 when Sir Stephen Paget recognized that tumor cells colonize certain organs preferentially based, in part, upon how they respond to signals from the microenvironment (Paget 1889). Trainer and colleagues later showed that treatment of murine melanoma cells with the DNA de-methylating agent 5-azacytidine resulted in reversible reduction of metastatic lung colonization (Trainer et al. 1988). Recent studies have shown that treatment of cells with 5-azacytidine can induce expression of the metastasis suppressor genes, Nm23 (Hartsough et al. 2001) and KAI1 (Sekita et al. 2001). Links

between metastasis and HDAC activity first became apparent when the breast cancer metastasis promoting gene, MTA1, was identified as a component of the NuRD:HDAC complex (Toh et al. 1994; Nicolson et al. 2003). MTA1 has subsequently been shown to repress estrogen-receptor-dependent transcription in an HDAC-dependent manner (Kumar et al. 2002). Likewise, loss of expression of heterochromatin protein 1 (HP1) has been associated with acquisition of metastatic potential in human breast cancer (Li et al. 2002b). Together, these findings support the hypothesis that regulation of the transcriptome by a variety of mechanisms is a critical determinant of cancer spread. The findings reported here represent the first direct evidence that a metastasis suppressor gene is a component of an HDAC complex. It is possible that specialized HDAC complexes may promote (as implied by MTA1) or inhibit (as implied by BRMS1) cancer metastasis. The data compel the hypothesis that metastasis is regulated, at least in part, by histone deacetylase activity, chromatin remodeling and/or transcriptional repression.

Connections between HDAC activity and cancer have emerged in recent years, stemming from observations that HDAC inhibitors, such as trichostatin A and SAHA, can induce growth arrest, differentiation, and/or apoptosis in transformed cultured cells (Marks et al. 2001). In pre-clinical animal models, HDAC inhibitors have demonstrated impressive anti-tumor activity which, in turn, led to several ongoing HDAC inhibitor clinical trials (Marks et al. 2001; Johnstone 2002; Vigushin and Coombes 2002; Kelly et al. 2002). The data presented here, along with data regarding MTA1 and HP1 cited above, are consistent with the hypothesis that HDAC inhibitors may influence not only primary tumors, but also distant metastases.

Interestingly, BRMS1 appears to be part of a protein family in which all of the characterized members are components of the mSin3:HDAC complex. During the original yeast two-hybrid screen, two cDNA clones identified as FLJ00052 were identified in the prostate library. As studies were underway to follow-up RBP1, mSin3 and HDAC findings, FLJ00052 was re-designated by GenBank as mSds3, the mammalian ortholog of *Saccharomyces cerevisiae* Suppressor of Defective Silencing 3 (Sds3). Sds3 has been implicated in gene silencing through a Sin3:Rpd3 pathway (Rpd3 in a yeast HDAC1 ortholog.); is an integral component of the yeast Sin3:Rpd3 complex; and is required for histone deacetylase activity (Lechner et al. 2000; Alland et al. 2002). BRMS1 shares 18% identity and 49% similarity with a large region of yeast Sds3 and 23% identity and 49% identity with mSds3. mSds3, anaologous to its yeast ortholog, is a

component of the mSin3:HDAC complex, stabilizes HDAC1 within the complex and augments HDAC activity (Alland et al. 2002). Another predicted mammalian protein of unknown function (designated MGC11296) is homologous to both Sds3 and BRMS1. Homology to BRMS1 is particularly strong (58% identity;79% similarity for the C-terminal 196 aa of BRMS1 and the N–terminal 196 aa of MGC11296). The high level of sequence similarity between these molecules, combined with their associations with mSin3:HDAC complexes, suggests the existence of a BRMS1 family of proteins that may play a crucial role in altering metastasis by regulating the so-called histone code (Berger 2002; Li et al. 2002b).

Although specific role(s) for BRMS1 within mSin3:HDAC complexes remain to be elucidated, several lines of evidence suggest that the metastasis suppressor may be involved in recruiting and stabilizing HDAC1 and/or modulating HDAC activity: (1) BRMS1 forms small complexes (~100 kDa and greater) with HDAC1, but forms only large complexes (~1.4 to 1.9 MDa) with Sin3B and SAP30 (Fig. 8B). (2) BRMS1 has distinct binding site(s) for the HDAC1:RbAp46/48 core subunit as compared to the rest of the complex (mSin3A, mSin3B, SAP30, HDAC2, RBP1), as demonstrated by BRMS1(Δ204-246) binding less effectively to HDAC1:RpAp46/48 than full-length BRMS1. In contrast, BRMS1(Δ204-246) binds the remaining complex components as effectively (Fig. 4). (3) The C-terminal 42 amino acids of BRMS1 appear to stabilize HDAC1:RbAp46/48 within the complex, as deletion of these residues specifically compromises binding to these three components (Fig. 4). (4) Both characterized BRMS1 family members (Sds3, mSds3) are required for optimal HDAC activity, and mSds3 specifically stabilizes HDAC1 within the mSin3 complex.

While remarkably similar in breast carcinoma and melanoma cell lines, BRMS1:mSin3:HDAC complexes were distinct. RbAp46 complexes with BRMS1 were not detected in MDA-MB-231 (Fig. 5), and the interaction with RbAp48 appeared less robust than in C8161.9 (compare Fig. 4 and Fig. 5). Differential binding of BRMS1(Δ204-246) to the HDAC1-RbAp46/48 subunit in C8161.9 was not observed in MDA-MB-231 (compare Fig. 4 to Fig. 5). At this juncture, it is not possible to distinguish whether the differences are due to cell origin or presence of mutations that abrogate interactions of RbAp46 with BRMS1:mSin3a. BRMS1-transfected MDA-MB-231 cells are suppressed for metastasis less than C8161.9 (40-90% *vs.* 90-100%). It is tempting to speculate that differences in metastasis suppression may be related to differential interaction

between BRMS1 and the HDAC1-RbAp46/48 subunit.

Preliminary data obtained with the BRMS1 deletion mutants reported here are consistent with a correlation between complexes involving BRMS1, mSin3 and HDAC and metastasis suppression. C8161.9.BRMS1(Δ164-246) and (Δ91-246) clones (mSin3 interactions severely impaired or lost Fig. 4)), fail to suppress metastasis (data not shown). However, more refined BRMS1 mutants will be required to determine if binding to the mSin3:HDAC complex is necessary for metastasis suppression. Systematic site-directed mutagenesis of BRMS1 coupled with metastasis assays are underway.

In summary, the metastasis suppressor BRMS1 is shown here to interact with enzymatically active mSin3:HDAC complexes. BRMS1 is also shown to form smaller complexes with HDAC1, and to repress transcription when recruited to a promoter region. Besides defining a milieu where BRMS1 works within cells, the data presented here imply that specific downstream mediators regulated, in part by HDAC activity, are critical to controlling metastatic behavior. Indeed, preliminary cDNA microarray and proteomic studies have identified a limited number of BRMS1-regulated genes (M. Cicek, R.S. Samant, M. Kinter, D.R. Welch and G. Casey, manuscript in preparation). Understanding the role(s) of BRMS1:mSin3:HDAC complexes in the regulation of gene expression promises to provide insights into metastasis suppression, HDAC-mediated chromatin regulation, and BRMS1 physiology in noncancerous cells.

Experimental Procedures

Cell lines, cell culture, and transfections

MDA-MB-231 is a human estrogen receptor- and progesterone receptor-negative cell line derived from a pleural effusion from an infiltrating ductal breast carcinoma. C8161 is a metastatic, amelanotic human melanoma cell line derived from an abdominal wall metastasis. C8161.9 is a highly metastatic clone obtained by limiting dilution cloning of C8161 (Welch et al. 1994). 66cl4 is a murine mammary carcinoma cell line derived from a spontaneous carcinoma in BALB/cfC3H mice (Miller et al. 1980; Aslakson and Miller 1992). All cell lines were cultured in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium (DME-F12), supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, Georgia), 1% non-essential amino acids, 1.0 mM sodium pyruvate. Transfected cells also

received 500 μg/ml G418 (Life Technologies Inc., Gaithersburg, Maryland). All cells were maintained on 100-mm Corning tissue culture dishes at 37°C with 5% CO_2 in a humidified atmosphere. MDA-MB-231 cells were passaged at 80-90% confluence using a solution of 0.125% trypsin and 2 mM EDTA in Ca^{2+}/Mg^{2+} free Dulbecco's phosphate buffered saline (CMF-DPBS). C8161.9 and 66cl4 cells were passaged at 80-90% confluence using 2 mM EDTA in CMF-DPBS. BRMS1 was cloned into the constitutive mammalian expression vector pcDNA3 (Invitrogen, San Diego, California) under control of the cytomegalovirus promoter. No antibiotics or antimycotics were used. All cell lines were found to be negative for *Mycoplasma spp.* contamination using a PCR-based method (TaKaRa, Madison, WI).

To detect BRMS1 protein expression, a chimeric molecule was constructed with an N–terminal epitope tag (SV40T epitope 901) (Kierstead and Tevethia 1993; Fu et al. 1996). Epitope-tagged full-length BRMS1 and deletion mutants were cloned into pcDNA3 before introduction into cells by electroporation (BioRad Model GenePulser, Hercules, California; 220 V, 960 μFd, ∞Ω). Briefly, cells (0.8 ml; 1 x 10^7 cells/ml) from 80% confluent plates were detached and plasmid DNA (10-40 μg) was added to the cells and the mixture placed onto ice for 5 min before electroporation, followed by 10 min on ice prior to plating on 100-mm cell culture dishes. Transfectants were selected using G418 (500 μg/ml). Single cell clones were isolated by limiting dilution in 96-well plates. Stable transfectants were assessed for protein expression by immunoblotting.

Constructs

Deletion mutants were created by unidirectional digestion with exonuclease III as previously described (Henikoff 1984). Briefly, pcDNA3 901-BRMS1 was digested by ApaI and Bsu36I in the 3' multiple cloning site, then digested with 150 units/pmol DNA exonuclease III (Promega) at 37°C. Reactions were stopped at different time points to create a nested set of C-terminal BRMS1 deletion mutants. Sequencing confirmed that 3' deletion mutants were successfully created: (1) 901-BRMS1(Δ204-246) + LFYSVT; (2) 901-BRMS1(Δ164-246) + TIL; and (3) 901-BRMS1(Δ91-246) + FYSVT. Additional amino acids were added because of a short stretch of vector DNA was transcribed prior to encountering a stop codon. Hereafter, these constructs will be designated BRMS1(Δ204-246), BRMS1(Δ164-246), and BRMS1(Δ91-246), respectively.

Antibodies

An antibody directed against the 901 epitope was generously provided by Dr. Satvir Tevethia.
Anti-MTA1 was a gift from Dr. Garth Nicolson. Anti-RBP1 (clone LY32 and initial aliquots of
clone LY11) were gifts of Dr. Philip Branton. Antibodies directed against HDAC1, HDAC3,
NCoR, RBP1 (clone LY11), SAP30, mSin3A, and SMRT were purchased from Upstate
Biotechnology (Lake Placid, NY, USA). Antibodies recognizing E2F and Rb were bought from
Pharmingen (San Diego, CA, USA). Antibodies directed against HDAC2, Mad1, Max, Mi-2,
p107, p130, RbAp46, RbAp48, SAP18, and mSin3B were obtained from Santa Cruz
Biotechnology.

Yeast Two-hybrid Screen

A yeast two-hybrid screen was performed to isolate cDNAs encoding BRMS1-interacting
proteins essentially as described in the manufacturer's instructions (Clontech MATCHMAKER
LexA). Full-length BRMS1 was cloned in-frame with the GAL4 DNA binding domain in the
pDBTrp (GIBCO-BRL/Invitrogen, Carlsbad, CA, USA) vector to obtain pDB-BRMS1. This
GAL4DB-BRMS1 fusion (bait) construct was used to transform AH 109 (*MATa, trp-901, leu2-
3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2,
URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ, MEL1*). Human breast, prostate and placenta cDNA libraries in
pACT2 (MATCHMAKER, BD Biosciences Clontech, Palo Alto, CA, USA) were screened in
yeast drop-out minimal medium lacking histidine, tryptophan and leucine. His$^+$ colonies were
tested for growth on minimal medium lacking adenine, tryptophan and leucine and
β-galactosidase activity as described previously (Van Aelst et al. 1993). cDNA plasmids were
isolated from each positive yeast clone using Zymoprep (Zymo Research, Orange, CA, USA)
and sequenced. The interaction phenotype was lost when either the bait or prey plasmid was lost
from the cell. Re-introduction of missing partners restored growth on minimal medium lacking
histidine, tryptophan and leucine, growth on medium lacking adenine, tryptophan and leucine,
and restoration of β-galactosidase activity.

^{35}S protein labeling

Cells were grown to 80-90% confluence in 100-mm tissue culture plates. Media was removed
and replaced with 3 ml of cysteine-methionine free media (GIBCO-BRL) containing 5 % FBS

for 1 hr. Media was removed and replaced with 3 ml of cysteine- and methionine-free media containing 5% FBS and 100 µCi/ml ^{35}S-express protein labeling mix (NEN). Cells were incubated for 18 hr before protein was collected for co-IP.

Co-Immunoprecipitation

Cells (90-95% confluence) were washed twice with ice-cold PBS and lysed with ice-cold lysis buffer (0.5% igepal CA-630 (Sigma), 50 mM Tris, pH 8.0, 150 mM NaCl, 2.0 mM EDTA) containing 1.0 mM PMSF, 2.0 µg/ml aprotinin, 50 mM NaF, 0.2 mM Na$_3$VO$_4$, and 10 µl/ml of a protease inhibitor cocktail containing 4-(2-aminoethyl) benzensulfonylfluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanido)butane (E-64), bestatin, leupeptin, and aprotinin (Sigma). Lysate was kept at 4°C during all subsequent steps. Lysate was passed through a 21 g needle several times, incubated on ice for 1 hour, then centrifuged for 1 hr at 12,000 x g in a Sorvall MC 12V microcentrifuge with an F12/M.18 rotor to remove insoluble debris. Lysates were then rocked gently in the presence of antibody for 1 hr, followed by the addition of 20 µl protein A/G PLUS agarose beads (Santa Cruz Biotechnology) and rocking overnight. Agarose beads were washed twice with ice-cold PBS, heated to 60°C in sample buffer, subjected to SDS-PAGE, and transferred to PVDF membrane for immunoblotting. For ^{35}S-labeled samples, films were exposed directly to PVDF membranes.

Size Exclusion Chromatography

Whole cell protein lysate (pooled from ten 100 mm plates using 1 ml lysis buffer each) was applied to a Superose 6 HR 10/30 size exclusion column (Amersham Pharmacia Biotech). The column was run using lysis buffer with 1.0 mM PMSF, 0.5 mM DTT at a flow rate of 0.2 ml/min. Fractions (500 µl) were collected and 420 µl of each fraction were used for co-IP. The remaining 80 µl was used for immunoblotting.

HDAC activity assay

Following co-IP, agarose beads were combined with 400 µl HDAC assay buffer (15 mM Tris, pH 7.9, 10 mM NH$_4$Cl. 0.25 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol) containing 1.5 µg ^{3}H-labeled chicken reticulocyte core histones (Kolle et al. 1998) with or without 250 mM sodium butyrate (an HDAC inhibitor). Samples were inverted continuously on a rotating wheel for 3 hr at 30°C, and HDAC activity was measured as described previously (Kolle et al. 1998).

Briefly, the reaction was stopped by adding 100 μl of 1M HCl /0.4 M acetic acid and 0.8 ml ethyl acetate. Samples were vortexed for 30 s and centrifuged at 8,000 x g for 5 min. An aliquot (0.6 ml) of the upper (organic) phase was then counted for radioactivity in 5 ml scintillation cocktail (Fisher).

Reporter assays

BRMS1 cDNA was cloned in-frame with N-terminal Gal4-DNA binding domain in pBIND (Promega). Subconfluent (80-90%) COS7 cells were transfected using the Fugene reagent (Roche Diagnostics Gmbh, Mannheim, Germany) with GAL4-BRMS1 fusion construct and a luciferase reporter plasmid containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, kindly provided by Dr. Ron Eisenman. pRLSV40 (Renilla luciferase) was used as a transfection control. Trichostatin A (50, 150, 300 ng/ml, Sigma) was added for 24 hr prior to lysis. Cells were lysed in Passive lysis buffer (Promega) 48 hr post-transfection. Cell extracts were assayed for luciferase activity using Dual-luciferase reporter assay system (Promega) and an automated luminometer Monolight™3010 (Pharmingen). Transfection efficiencies were normalized using the Renilla luciferase control.

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Fig. 1. Yeast two-hybrid and co-IP indicate that BRMS1 interacts with retinoblastoma binding protein 1 (RBP1): *A,* Growth of representative BRMS1 interacting protein candidates on minimal media lacking histidine, tryptophan and leucine. *B,* Growth of representative BRMS1 interacting protein candidates on minimal medium lacking adenine, tryptophan and leucine. + indicates positive control, - indicates negative control (AH109 with BRMS1 or interactor cDNA alone). *C,* BRMS1 co-IP RBP1 from whole cell lysate (1 mg) in MDA-MB-231 cells. Anti-901 was used to IP epitope-tagged BRMS1, and also pulled down RBP1, as shown by Western blot. Anti-901 did not pull down RBP1 in vector-transfected cells. *D,* BRMS1 co-IP RBP1 from whole cell lysate (1 mg) in C8161.9 cells. Anti-901 was used to IP epitope-tagged BRMS1, and also pulled down RBP1, as shown by Western blot. Anti-901 did not pull down RBP1 in vector-transfected cells, and anti-Lamin A/C (an irrelevant antibody) did not pull down RBP1 in BRMS1-transfected cells. *E,* Anti-RBP1 co-IP BRMS1 in MDA-MB-231 cells. Immunoblotting with α-901 was used as a positive control, and α-Lamin A/C was used as a negative control. *F,* Anti-RBP1 co-IP BRMS1 in C8161.9 cells. Immunoblotting with α-901 was used as a positive control, and α-mSin3A and α-SAP18 were used as negative controls.

Fig. 2. Binding to RBP1 is abrogated as C-terminal amino acids are removed from BRMS1 and BRMS1 does not co-IP Rb or p107: *A,* Whole cell lysates (1 mg) were prepared from MDA-MB-231 cells expressing 901-epitope-tagged BRMS1 or BRMS1(Δ204-246) (see Fig. 2C). BRMS1(Δ204-246) co-IP RBP1 (lane 3). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1). To determine relative protein expression, 50 μg of protein lysate from each transfected construct was immunoblotted (lanes 4-6) (the exposure for α-901 shown here was not long enough to show expression in lanes 4 and 5). BRMS1 did not co-IP Rb or p107. *B,* Whole cell lysates (1 mg) were prepared from C8161.9 cells expressing BRMS1 and BRMS1 deletion mutants (see Fig. 2C) with protein levels comparable to the clone expressing full length BRMS1. The deletion mutants exhibited varying abilities to co-IP the above-mentioned proteins (lanes 4-6). Anti-901 (lane 1) and an irrelevant antibody (anti-Lamin A/C, lane 2) did not pull down RBP1 in vector-transfected cells. *C,* Schematic of BRMS1 deletion mutants. > indicates IgG light chain.

Fig. 3. BRMS1 co-IP several proteins using ^{35}S-labeled whole cell lysates: Using radiolabeled protein lysate from MDA-MB-231 cells, anti-901 was used to immunoprecipitate epitope-tagged

BRMS1. Immunoprecipitation of BRMS1 revealed at least twelve co-immunoprecipitated proteins. Arrows with numbers indicate co-immunoprecipitated proteins and approximate MWs in kDa.

Fig. 4. BRMS1 co-IP at least seven members of the mSin3 HDAC complex in C8161.9 human melanoma cells: *A,* BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp46, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected C8161.9 cells (lane 3). Whole cell lysates (1 mg) were also prepared from C8161.9 cells expressing BRMS1 deletion mutants (see Fig. 2C) with protein levels comparable to the clone expressing full-length BRMS1. Deletion mutants exhibited varying abilities to co-IP the above-mentioned proteins (lanes 4-6). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1), and anti-Lamin A/C (an irrelevant antibody) did not pull down these proteins in BRMS1-transfected cells (lane 2). > indicates IgG light chain.

Fig. 5. BRMS1 co-IP at least six members of a mSin3 histone deacetylase co-repressor complex in MDA-MB-231 human breast carcinoma cells: *A,* BRMS1 co-immunoprecipitated mSin3A, mSin3B, HDAC1, HDAC2, RbAp48, and SAP30 from whole cell lysates (1 mg) of stably transfected MDA-MB-231 cells (lane 2). Whole cell lysates (1 mg) were also prepared from MDA-MB-231 cells expressing BRMS1 deletion mutant (Δ204-246) (see Fig. 2C) with protein levels comparable to the clone expressing full length BRMS1. BRMS1 (Δ204-246) also co-immunoprecipitated the above-mentioned proteins (lane 3). Anti-901 did not pull down these proteins in vector-transfected cells (lane 1). To determine relative protein expression, 50 μg of protein lysate from each transfected construct was immunoblotted (lanes 4-6) (the exposure for α-901 was not long enough to show expression in lanes 4 and 5).

Fig. 6. HDAC1, HDAC2, SAP30, RBP1, mSin3B, and mSin3A co-IP BRMS1: *A,* In BRMS1-transfected C8161.9 cells, antibodies recognizing mSin3A, mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell lysate. Antibodies directed against SAP18, RbAp46, RbAp48, and pRb did not co-IP BRMS1. Anti-901 was used as a positive control. * In the bottom panel, increased exposure time was used to reveal co-immunoprecipitated BRMS1, causing a cross-reacting band of slower mobility to become visible. *B,* In BRMS1-transfected MDA-MB-231 cells, antibodies directed against mSin3B, SAP30, HDAC1, HDAC2, and RBP1 co-immunoprecipitated BRMS1 from 1 mg of whole cell

lysate. Antibodies directed against mSin3A, SAP18, RbAp46, RbAp48, and pRb did not co-IP BRMS1. Anti-901 was used as a positive control.

Fig. 7. BRMS1 pulls down HDAC activity: Whole cell lysate (6 mg total protein) was prepared from BRMS1-transfected C8161.9 cells as well as from C8161.9 cells expressing BRMS1 deletion mutants (Δ204, Δ164, and Δ91) and vector-transfected (V) cells. Anti-901 was used to immunoprecipitate BRMS1 and BRMS1 deletion mutants from this lysate, and co-immunoprecipitated HDAC activity was measured. The HDAC inhibitor sodium butyrate (250 mM) was used to show that release of ^{3}H-acetyl groups was specifically due to HDAC activity. Anti-HDAC1 was used as a positive control (*10μg anti-HDAC1 used, **5 μg anti-HDAC1 used). Anti-Lamin A/C was used as a negative control. Bars with error bars represent mean plus standard error for 2 independent experiments. See Figure 2C for a schematic of the BRMS1 deletion mutants.

Fig. 8. BRMS1 co-IP a large (~1.6 MDa) complex containing HDAC1, SAP30, and mSin3B, as well as smaller complexes containing HDAC1: *A,* Elution profile of BRMS1, HDAC1, SAP30, and mSin3B in BRMS1-transfected C8161.9 cells. Whole cell lysate (3 mg total protein) was prepared and applied to a Superose 6 size exclusion column. Fractions (500 μl) were collected and 20 μl of each fraction were subjected to SDS-PAGE and immunoblotting. *B,* Immunoprecipitation of BRMS1 within eluted fractions. Whole cell lysate (3 mg total protein) was prepared from BRMS1-transfected C8161.9 cells and applied to a Superose 6 size exclusion column. Fractions (500 μl) were collected and anti-901 was used to immunoprecipitate BRMS1 from 420 μl of each fraction. Immunoprecipitated complexes were subjected to PAGE and immunoblotting.
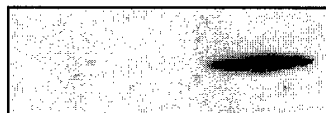
Fig. 9. BRMS1 represses transcriptional activity in vivo. Using a luciferase reporter assay containing four GAL4 binding sites upstream of the myelomonocytic growth factor minimal promoter, BRMS1 strongly repressed (~80%) basal transcription compared to the pBIND vector alone.
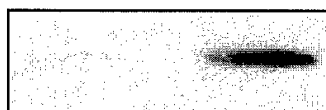
A
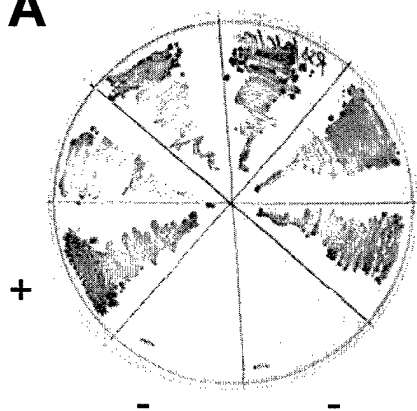
B

+ −   + −
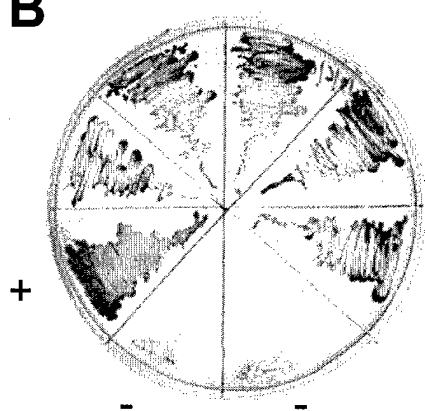
C Construct: Vector   BRMS1

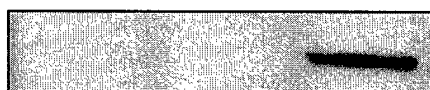IP: 901   901   WB:

RBP1

BRMS1
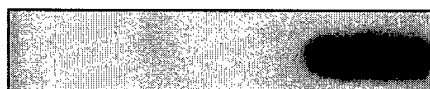
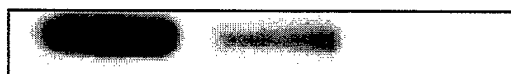**D** Construct: Vector   BRMS1
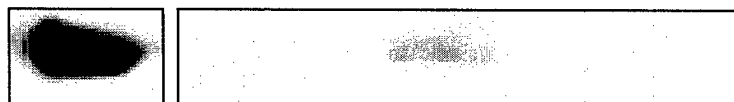
IP: 901   Lamin   901   WB:
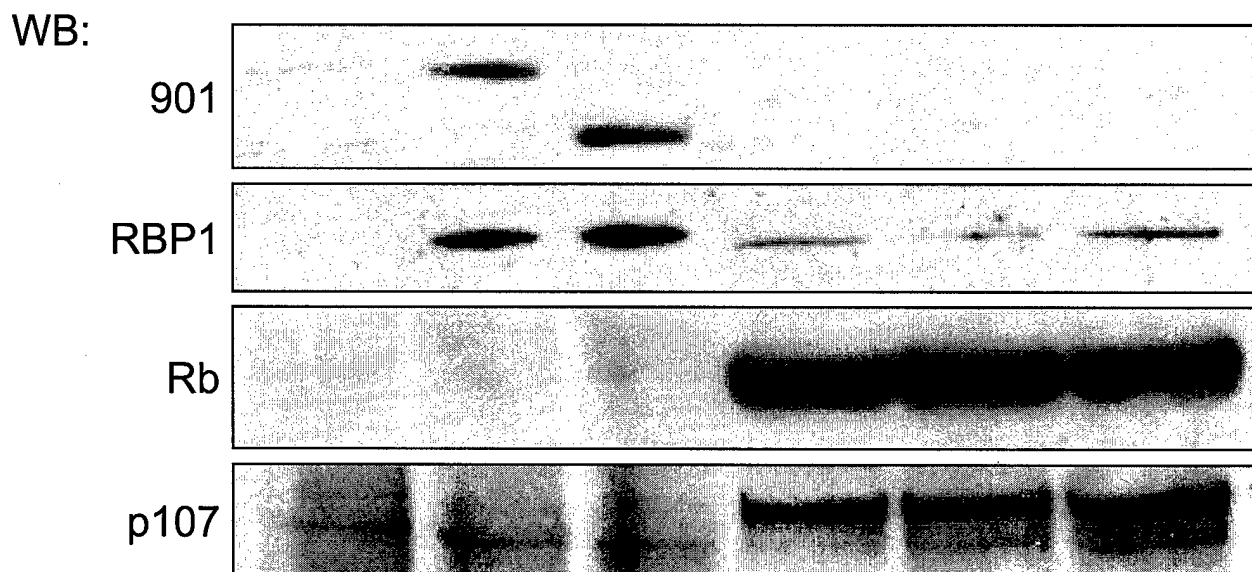
RBP1

BRMS1

E   IP: 901   RBP1   Lamin

WB:α901

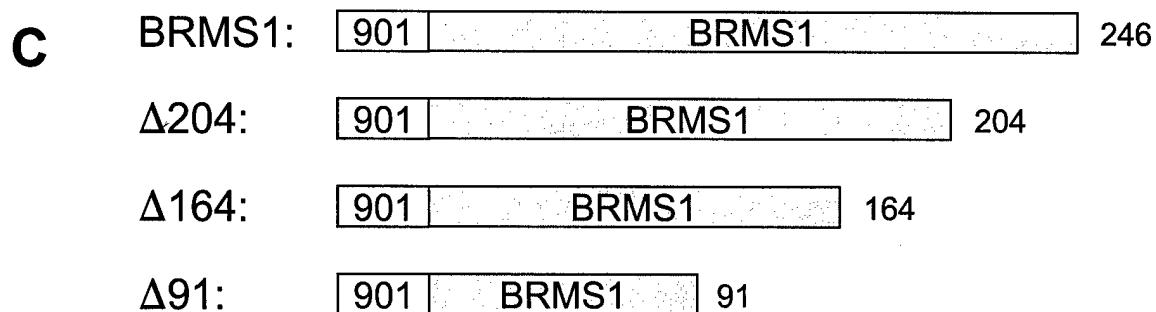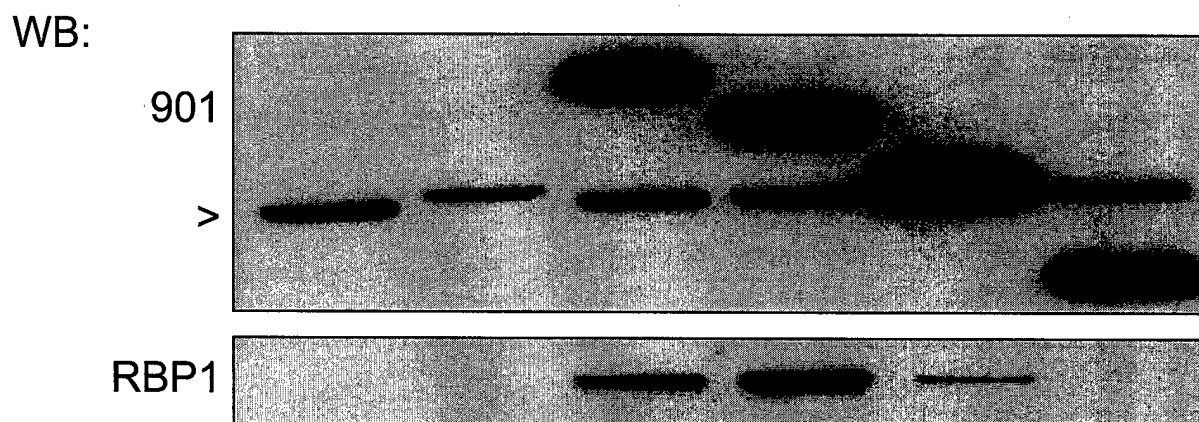**F**   IP: 901   mSin3A   RBP1   SAP18
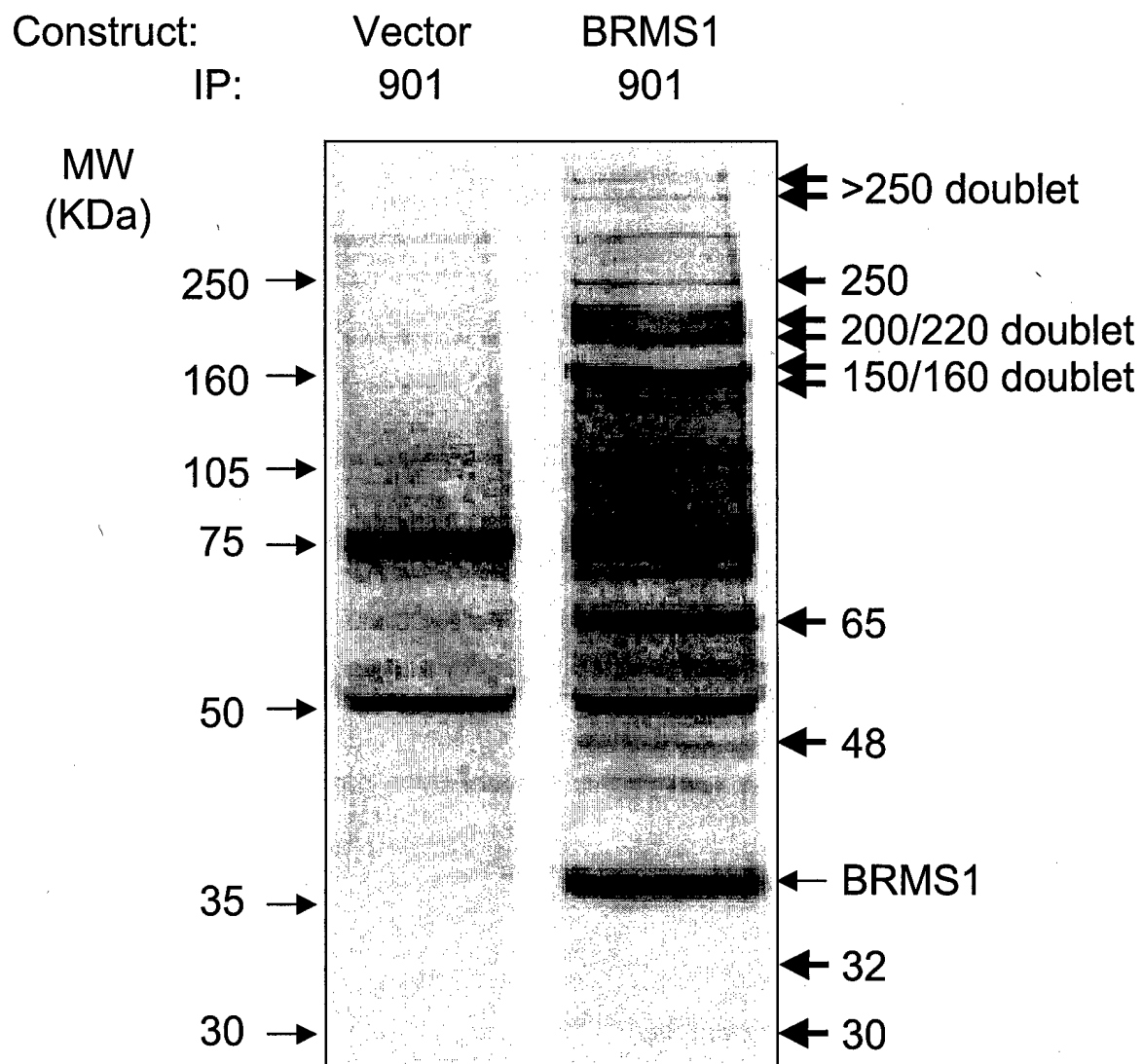
WB:α901

A

Lane:	1	2	3	4	5	6
Construct:	Vector	BRMS1	Δ204	Vector	BRMS1	Δ204
IP:	901	901	901	none	none	none

WB:

901

RBP1

Rb

p107

B

Lane:	1	2	3	4	5	6
Construct:	Vector	BRMS1	BRMS1	Δ204	Δ164	Δ91
IP:	901	Lamin	901	901	901	901

WB:

901

>

RBP1

C

BRMS1: | 901 | BRMS1 | 246

Δ204: | 901 | BRMS1 | 204

Δ164: | 901 | BRMS1 | 164

Δ91: | 901 | BRMS1 | 91

Construct: Vector BRMS1
IP: 901 901

MW (KDa)

>250 doublet
250 → 250
200/220 doublet
160 → 150/160 doublet
105 →
75 →
65
50 → 48
35 → BRMS1
32
30 → 30

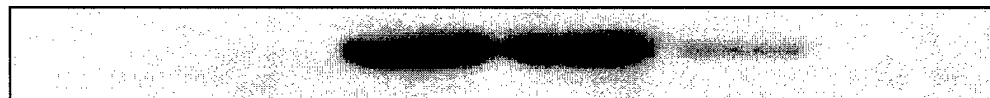| Lane:	1	2	3	4	5	6
Construct:	Vector	BRMS1	BRMS1	Δ204	Δ164	Δ91
IP:	901	Lamin	901	901	901	901

WB:

901

\>

mSin3A

mSin3B

SAP30

HDAC1

HDAC2

RbAp46

RbAp48

Lane:	1	2	3	4	5	6
Construct:	Vector	BRMS1	Δ204	Vector	BRMS1	Δ204
IP:	α901	α901	α901	none	none	none

WB:

αmSin3A

αmSin3B

αHDAC1

αHDAC2

αSAP30

αRbAp48

αRbAp46

αSAP18

A

IP: **901**   **mSin3A** **RBP1** **SAP18**

WB: 901

IP: **RbAp46** **RbAp48** **mSin3A** **SAP30**

901

IP: **Lamin** **Rb** **HDAC1**

901

IP: **HDAC2** **mSin3A** **mSin3B** **RbAp46**

901

*

B

IP: **901** **RBP1** **Lamin**

WB: 901

IP: **mSin3B** **mSin3A** **HDAC1** **HDAC2**

901

IP: **RbAp48** **RbAp46** **SAP30**

901

HDAC activity;cpm-released radioactivity

| 2800 | 2600 | 1400 | 1200 | 1000 | 800 | 600 | 400 | 200 | 0 |

Butyrate: –   +   –   +   –   +   –   +   –   +   –   +   –   +

Construct: BRMS1   BRMS1   V   BRMS1   Δ204   Δ164   Δ91   BRMS1

Antibody: αHDAC1* αHDAC1** α901   α901   α901   α901   α901   αLamin A/C